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On the variability of spore ornamentation in *Laccaria tortilis* (*Basidiomycota*, *Agaricales*)

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Abstract — "*Laccaria tortilis*" sensu Clémençon 1984 is described as the new form, *Laccaria tortilis* f. *clemenconii,* based on material from Kamchatka (Asiatic Russia), Switzerland, Austria, and Italy. ITS rDNA analysis indicates that the new form is molecularly identical to *Laccaria tortilis,* although smaller spores with shorter spines distinguish it morphologically. Scanning electronic micrographs of the spores and a dichotomous key to European *Laccaria* species with mono-bisporic basidia, are provided.

Key words — *Agaricomycetes*, *Hydnangiaceae,* taxonomy, biodiversity

Introduction

Laccaria tortilis (Bolton) Cooke is the smallest representative of the genus *Laccaria* Berk. & Broome. The species is widespread, known from Europe (Mediterranean zone included) and both North and South America (Mueller 1992). Its tiny basidiomata usually grow in groups on poor, bare, and very moist soil and are often found along the course of rivers and streams. It is easily recognized due to its strongly plicate-striate and deformed pileus, very broad lamellae, large globose spores [(9.2–)10–14.5(–16) × (8.3–)10–14.5(–16) µm] covered with very crowded, conic to pyramidal echinulae that are 1.4–3.2(–4) µm long and ≤ 2.3 µm wide at the base (see Mueller 1992: Figs 29a, 55c–d; Vesterholt 2008: 659, Fig. C), and mono-bisporic basidia (Vellinga 1995; Mueller 1987, 1992; Cacialli et al. 1996; Vesterholt 2008).

Mueller designated a collection by P.D. Orton at Tanfield Lodge, Scotland, on 6.9.1969, as epitype for *L. tortilis* (Mueller 1997). Mueller (1987) had previously described the collection as a neotype based on its globose spores averaging 13.4 × 13.4 µm ["11.5–14.7(–17) × 11.5–14.7(–17) µm"] and echinulae "2.3–4 µm" long and "1.3–1.8 µm" wide at the base (see Mueller 1987: Fig. 2a).

In his key to the European *Laccaria*, Clémençon (1984) seems to have reported a different taxon under the name "*Laccaria tortilis*", which is distinguished from the above collection by slightly smaller spores bearing much shorter echinulae, only "1–1.5" µm long. Clémençon (1984) referred the species with elongate echinulae to the name "*Laccaria echinospora* (Speg.) Singer", a species concept followed by (among others) Kalamees & Vaasma (1993) and Pázmány (1992, 1994). However, *Agaricus echinosporus* Speg. should be regarded as a heterotypic synonym of *Agaricus tortilis* Bolton because Spegazzini's original material is conspecific with the type collection of *Laccaria tortilis* (Mueller 1992).

During fieldwork in Kamchatka (Asiatic Russia), K. Kalamees and M. Vaasma had the opportunity to collect very small specimens of a *Laccaria* whose micromorphological features fit Clémençon's and Pázmány's concepts of *Laccaria tortilis* rather well. The basidiomata were found in Kronok Nature Reserve, Uzon Caldera on 21.8.1978, growing on a clayey moist grassland area with pebbles, covered with mosses and sparse herbs. Kalamees & Vaasma (1993) described this collection, characterized by globose spores with 1.3(–1.6) µm long echinulae under the name "*Laccaria tortilis* (Bolt.) Boud. sensu Clémençon, Z. Mykol. 50(1): 7, 1984", thus noting that Clémençon's concept did not represent the typical *L. tortilis*.

While revising the *Laccaria* species housed in TAAM, we ascertained that the taxon collected in Uzon Caldera by Kalamees and Vaasma (TAAM 120146) represents the same taxon as *Laccaria tortilis* sensu Clémençon (1984), which apparently differed from the *Laccaria tortilis* concept typified by Mueller (1997).

Additional basidiomata representing the small-spored taxon were collected by Enzo Musumeci, Irmgard Greilhuber, and Marco Contu from Switzerland, Austria, and Sardinia (Italy), respectively.

In the present work we propose, for the pseudonomen "*Laccaria tortilis*" sensu Clémençon, a new form, *Laccaria tortilis* f. *clemenconii*, named after Heinz Clémençon and typified by a Swiss collection.

Materials & methods

Morphology

Macro- and micromorphological descriptions are based on both fresh and dried material; dried material was reinflated with 3% KOH and mounted in Phloxin B in order to detect the spore ornamentation. Additional data from the description published by

Clémençon (1984) are reported in brackets. Spore size (excluding ornamentation) is expressed both as a range and mean value based on 30 randomly chosen spores. Author citations follow the Index Fungorum website (http://www.indexfungorum.org/Names/AuthorsofFungalNames.asp).

Herbarium abbreviations follow Thiers (2011). The type material is currently kept in TO.

SEM photographs

Electronic micrographs were made under a Zeiss DSM 950 SEM following Moreno et al. (1995).

DNA extraction, PCR amplification, and DNA sequencing

Genomic DNA was isolated from 1 mg of 4 herbarium specimens (Tab. 1) using the DNeasy Plant Mini Kit (Qiagen, Milan Italy) according to the manufacturer's instructions. Universal primers ITS1f/ITS4 were used for the ITS region amplification (White et al. 1990; Gardes & Bruns 1993). Amplification reactions were performed in PE9700 thermal cycler (Perkin-Elmer, Applied Biosystems) in a 25 µl reaction mixture using the following final concentrations or total amounts: 5 ng DNA, 1× PCR buffer (20 mM Tris/HCl pH 8.4, 50 mM KCl), 1 µM of each primer, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.5 unit of *Taq* polymerase (Promega). The PCR program was as follows: 3 min at 95°C for 1 cycle; 30 s at 94°C, 45 s at 50°C, 2 min at 72°C for 35 cycles, 10 min at 72°C for 1 cycle. PCR products were resolved on a 1.0% agarose gel and visualized by staining with ethidium bromide. PCR products were purified and sequenced by DiNAMYCODE srl (Turin, Italy). Sequence assembly and editing were performed using Geneious v5.1.6 (Drummond et al. 2009). The sequences are deposited in GenBank under the accession numbers given in Table 1.

Sequence alignment and phylogenetic analysis

Sequences included in the phylogenetic analyses were either generated in this study (Tab. 1) or retrieved from GenBank. Multiple sequence alignments for ITS fragments

Table 1. *Laccaria tortilis* samples used in this molecular study.

Species	Country (locality)	Herbarium (ITS) acc. nos. date — collector	Habitat
1—**L. tortilis* f. *clemenconii*	Switzerland (Kanton Basel, St. Chrischona)	TO-AVHL14 (JF284353) 25.04.2008 — E. Musumeci	Under *Picea abies*
2—**L. tortilis* f. *clemenconii*	Switzerland (Kanton Basel, St. Chrischona)	TO-AVHL15 holotype (JF284354) 24.05.2008 — E. Musumeci	Under *Picea abies*
3—**L. tortilis* f. *clemenconii*	Switzerland (Kanton Solothurn, Rodersdorf)	TO-AVHL17 (JF284355) 06.08.2008 — E. Musumeci	Near *Fagus sylvatica, Quercus*
4—**L. tortilis* f. *clemenconii*	Italy (Sardinia,Olbia-Tempio, Mt. Limbara, Vallicciola)	AH38997 (JF284356) 22.06.2008 — M. Contu	Stream bank
5— *L. tortilis*	USA: Colorado (Boulder Co. near Hwy. 72)	DBGH20904 (DQ149872) 23.06.2000 — [Osmundson et al. 2005]	Under *Salix*, *Alnus*

* = newly sequenced samples.

were generated using ClustalX 2.0 (Larkin et al. 2007) with default conditions for gap opening and gap extension penalty. The alignment was slightly edited using MEGA 4.0 (Tamura et al. 2007). Phylogenetic analyses were performed using both Bayesian Inference (BI) and Maximum Likelihood (ML) approaches. The BI was performed with MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001) with four incrementally heated simultaneous Monte Carlo Markov Chains (MCMC) ran over 10 millions generations, under GTR+Γ evolutionary model. Trees were sampled every 1000 generations resulting in an overall sampling of 10,001 trees; the first 2500 trees were discarded as "burn-in" (25%). For the remaining trees, a majority rule consensus tree showing all compatible partitions was computed to obtain estimates for Bayesian Posterior Probabilities (BPP). Branch lengths were estimated as mean values over the sampled trees. ML was performed with RAxML (Stamatakis et al. 2005) under GTRGAMMA model and using thorough bootstrap with 20 runs and 1000 replicates. In both analyses a *Laccaria laccata* sequence (Genbank accession FJ845416) was used as outgroup.

The ML consensus tree was used merely for comparison with the Bayesian tree and to support the analysis. However the ML bootstrap and BPP (Bayesian posterior probability) values over 50% are reported in the resulting tree.

Results

Molecular results

Maximum likelihood and Bayesian inferences were performed on a total of 19 samples, including 15 sequences available from GenBank and four newly sequenced specimens. Final alignment length was 634 bp. Both maximum likelihood and Bayesian analyses resulted in the same topology (Fig. 1). In both analyses the four "*L. tortilis*" sensu Clémençon sequences clearly cluster with the *L. tortilis* sequence (the tortilis clade) with 100 percent of both BPP and ML bootstrap values. Taking into account the robustness of the clade, the five sequences can be considered conspecific.

Taxonomy

Laccaria tortilis f. ***clemenconii*** Contu, Vizzini, Kalamees & G. Moreno, **f. nov.**

MycoBank MB519740

"*Laccaria tortilis*" sensu Clémençon (1984: 7), Kalamees & Vaasma (1993: 126), Pázmány (1994: 8) non *Laccaria tortilis* (Bolton) Cooke, Grevillea 12: 70 (1884) [Mueller (1987: 306)]

A typo differt habitu regulariore, sporis minoribus spinisque sporalibus (1–) 1.5–2(–2.5) µm longis.

Type — Switzerland, Kanton Basel, St. Chrischona, 480 m a.s.l., 24.V.2008, leg. E. Musumeci (**holotype** TO AVHL15).

Etymology — named in honour of Heinz Clémençon, leading specialist of *Laccaria* and other *Agaricales*.

Pileus 8–25 mm wide, not fleshy, convex, becoming plane to uplifted, often depressed at the centre, plicate-striate, margin often undulate-distorted,

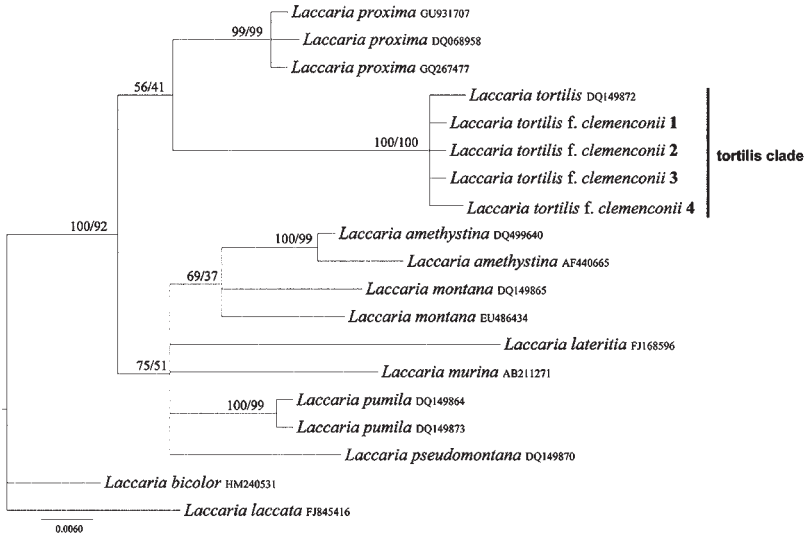

FIGURE 1. Bayesian tree (GTR+G, 10mil-gen, burn-in 25%); BPP values and MLB values (_/_). Numbers (1–4) refer to the collections reported in TABLE I. The bar indicates number of substitutions per site.

fulvous to salmon-pink or pale pink, fading buff, finely fibrillose. LAMELLAE distant, thick, very broad, sinuate to adnate, occasionally subdecurrent to decurrent, pink, white-pruinose in old basidiomata. STIPE 8–25 × 1–2(–3) mm, rather short, gracile, mostly equal, polished or slightly fibrillose-sericeous, concolorous with the pileus, basal mycelium white. CONTEXT very scarce, pink, darker towards the stipe base, unchanging. SMELL indistinct to agreeable, at times fruity. TASTE indistinct. SPORE-PRINT white.

SPORES 10–12(–15) × 10–12(–14.5) µm, mostly 11 × 11 µm, globose, hyaline, thick-walled, strongly echinulate, echinulae very crowded, pyramidal, (1–)1.5–2(–2.5) µm long, mostly 1.5 µm long, 1–1.5 µm wide at the base, apex acute (FIG. 2a–f). BASIDIA 45–60 × 9–12 µm, clavate, stout, mostly monosporic or bisporic, rarely intermixed with scarce tri- or tetrasporic ones, with basal clamp-connection; SUBHYMENIUM ramose; HYMENOPHORAL TRAMA regular, made up of cylindrical, subhyaline 4.5–15 µm wide hyphae. MARGINAL CELLS scarce, filamentous or subclavate, 4–6 µm wide, hyaline, thin-walled. PILEUS SURFACE consisting of a poorly differentiated cutis made up of rather compact, cylindrical hyphae, 4.5–12 µm wide, rarely interwoven and with scattered fascicles of perpendicular hyphae, with a pale brown intraparietal and plasmatic pigment. CLAMP-CONNECTIONS abundant.

Habitat and distribution. Gregarious on clayey moist areas covered by mosses, often in small fascicles of 3–5 basidiomata. Autumn. Known from Kamchatka (Asiatic Russia), Switzerland, Austria, and Sardinia (Italy).

Additional material studied: **RUSSIA**, "Fungi Kamtschatici", *Tricholomataceae*, *Laccaria tortilis* (Bolt.) S.F.Gray ss Moser 1978, Regio kamtschatica, Kronoki looduskaitseala, Uzoni kaldeera, jõelamm, 21.8.1978, leg. K. Kalamees, det. M. Vaasma, TAAM 120146 (rev. a K. Kalamees 5.11.1992 atque rinominata *Laccaria tortilis* ss Clémençon)"; **SWITZERLAND**: **Kanton Basel**, St. Chrischona, 460 m a.s.l., 25.04.2008, leg. E. Musumeci, (TO AVHL14); **Kanton Solothurn**, Rodersdorf, 06.08.2008, leg. E. Musumeci (TO AVHL17); **AUSTRIA**: Braunau am Inn district, St. Peter am Hart community, Hartwald, 370 m a.s.l., 20.09.2009, leg. R. Krisai (TO AVHL22 & WU); **ITALY**: **Sardinia**, Olbia-Tempio, Mount Limbara, Vallicciola, 22.06.2008, leg. M. Contu (AH 38997).

Discussion

The spore ornamentation made up of very crowded pyramidal echinulae, 1.0–1.5 µm wide at the base, the strictly globose spores, and the mono-bisporic basidia undoubtedly place *Laccaria tortilis* sensu Clémençon, Pázmány, and Kalamees & Vaasma within the *L. tortilis*-complex. As pointed out in the introduction, the new form and forma *tortilis* are readily differentiated based on spore size and ornamentation. Nonetheless, molecular studies (Fig. 1) show that they represent a single species with rather variable spore ornamentation. According to Mueller (1992), *Laccaria tortilis* var. *gracilis* Peck is synonym of *L. ohiensis* (Mont.) Singer. There is another small-sized bisporic *Laccaria* producing spores with equally crowded echinulae — *L. pumila* Fayod (= *L. altaica* Singer; but see Sivertsen 1993 and Vesterholt 2008) — but it is separated from *L. tortilis* by its ellipsoid spores with much shorter echinulae (usually 0.5–0.8(–1) µm long; Trimbach 1978, Bon 1983, Ballero & Contu 1989, Mueller 1992, Contu 2003) and ITS sequences (Fig. 1). Another small-sized species, *L. nana* Massee, differs in dark cinnamon colouration, a non-striate pileus, and globose spores with echinulae measuring (2.3–)2.8–4 µm long and 1.8–2.3 µm wide at the base (Mueller 1992). In *Laccaria*, at least one infraspecific taxon has been described with spores bearing shorter spines than those of the type: *Laccaria masoniae* var. *brevispinosa* McNabb, from New Zealand, has echinulae 1.2–1.5 µm long, while the echinulae are 2–3.5 µm long in *L. masoniae* G. Stev. var. *masoniae* (McNabb 1972). These last two taxa, not known from Europe, clearly differ from *L. tortilis* in their violaceous tinged basidiomata. Infraspecific variants characterised by spore echinulae longer than those of the type have been described elsewhere, such as *Laccaria macrocystidiata* var. *longispinosa* Contu (Contu 2003). Therefore, we do not regard spore ornamentation as a good taxonomic marker, at least in some cases, to delimit species concepts in *Laccaria*.

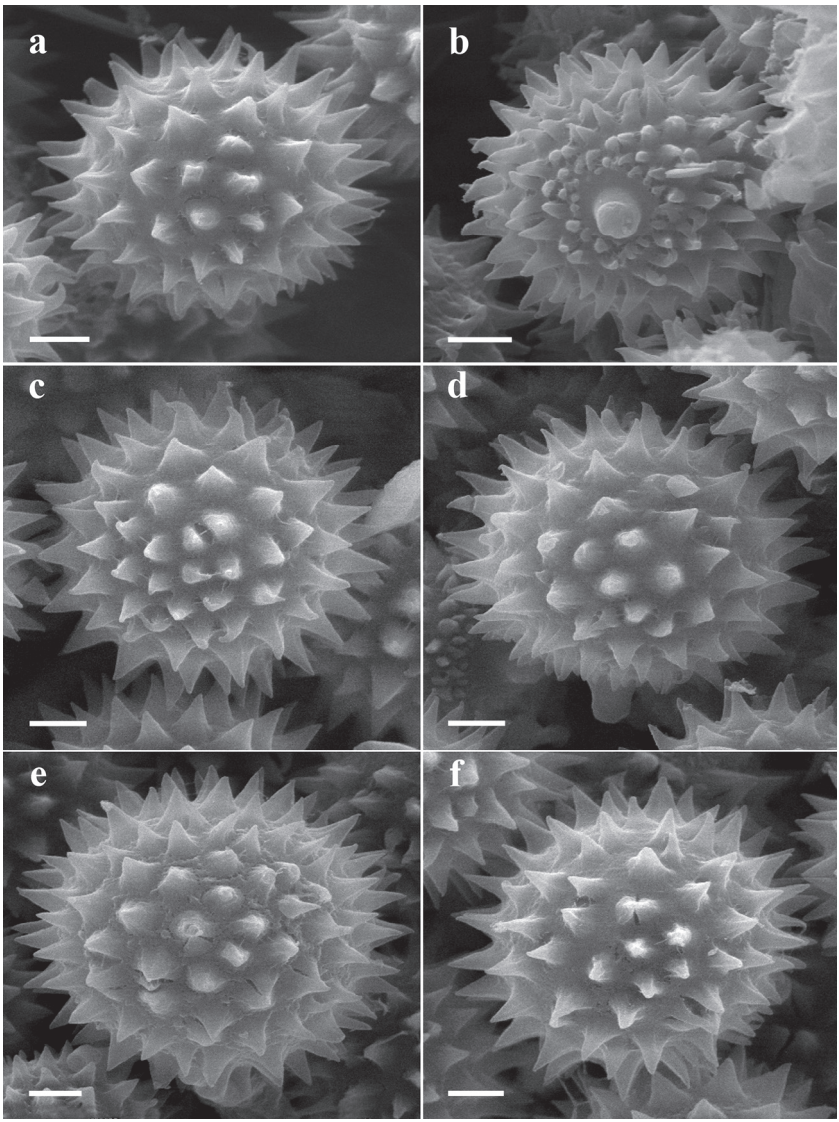

Figure 2. *Laccaria tortilis* f. *clemenconii*. Spores (SEM photographs). **a–b**. Collection TO AVHL15 (Switzerland, holotype). **c–d**. Collection TO AVHL17 (Switzerland). **e–f**. Collection AH 38997 (Italy). Bars = 2 µm.

Key to the European bisporic species of the genus *Laccaria*

1 Mycelium at stipe base violaceous; in moist areas among bog mosses (rare, known only from Spain) ..... ***L. violaceibasis*** Contu & Fernández Sas.
1 Mycelium at stipe base white 2
2 Basidiomata reddish, with evident lilac hues, odour pronounced, fruity (montane) ..... ***L. vinaceorosea*** Contu
2 Basidiomata lacking lilac hues; odour not fruity 3
3 Spores < 11 µm on average 4
3 Spores larger, ≥ 11 µm on average 5
4 Spore echinulae 1.3–1.8–2 µm long, quite spaced, pyramidal; pileus scaly-areolate, striate only towards the margin, stipe fibrillose-striate; montane woods..... ***L. bisporigera*** Contu & Ballero
4 Spore echinulae 0.8–1 µm long, conical, crowded; pileus almost smooth, strongly striate, stipe slightly fibrillose; mainly in Mediterranean areas, with *Acacia*, *Eucalyptus* and *Cupressus* ..... ***L. lateritia*** Malençon
5 Spores subglobose, broadly ellipsoid to oblong 6
5 Spores perfectly globose..... 7
6 Spores 9.5–15 × 8–12 µm, echinulae 0.5–0.8 µm long ..... ***L. pumila***
6 Spores 10–12 × 9–10 µm, echinulae 0.5–1 µm long ..... "***L. vinaceorosea***" [forma ined.]
7 Spores 13 µm on average, with echinulae ≤ 3.2(–4) µm long and ≤ 2.3 µm wide at the base..... ***L. tortilis*** f. ***tortilis***
7 Spores 11 µm on average, with echinulae ≤ 2(–2.5) µm long and ≤ 1.5 µm wide at the base..... ***L. tortilis*** f. ***clemenconii***

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